

**ON DIFFERENT ACTIVATION MECHANISMS
OF THE HUMAN COMPLEMENT SYSTEM**

by
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To Anna, Malin and Johan



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The thesis is based on the following publications. They will be referred to by their Roman numerals:

- I Johnson, U., Kamme, C., Laurell, A.-B. & Nilsson, N.I.
C1 subcomponents in acute pneumococcal otitis media in children.
Acta path. microbiol. scand. Sect. C, 85, 10-16, 1977
- II Laurell, A.-B., Johnson, U., Mårtensson, U. & Sjöholm, A.G.
Formation of complexes composed of C1r, C1s and C1 in-
activator in human serum on activation of C1.
Acta path. microbiol. scand. Sect. C, 86, 299-306, 1978
- III Johnson, U.
The role of C1s and C1r and properdin in the initiation
of the C3b dependent feedback mechanism of the complement
system.
Acta path. microbiol. scand. Sect. C, 86, 73-78, 1978
- IV Johnson, U. & Prellner, K.
Purification of C-reactive protein on DEAE-cellulose by a
simple two-step procedure utilizing the calcium-dependency
of the protein.
Biochimica et Biophysica Acta, 494, 349-353, 1977
- V Johnson, U.
Properdin acting as a C3 convertase.
Acta path. microbiol. scand. Sect. B, 82, 914-916, 1974
- VI Johnson, U. & Laurell, A.-B.
Activation of complement in anaphylactoid reactions in
connection with infusion of dextran.
Scand. J. Immunol. 3, 673-676, 1974
- VII Johnson, U. & Laurell, A.-B.
Complement activation in meningococcal septicemia.
Acta path. microbiol. scand. Sect. C, 83, 285-288, 1975
- VIII Johnson, U., Ohlsson, K. & Olsson, I.
Effects of granulocyte neutral proteases on complement
components.
Scand. J. Immunol. 5, 421-426, 1976

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INTRODUCTION

The complement system. The complement system constitutes about 4 per cent of the total plasma protein. Activation elicits biological functions essential for the normal immune defence and for the inflammatory reaction.

During the last decade many of the complement components have been isolated in highly purified form and characterized. Most of them have been found to be glycoproteins, some of which are zymogens. All of the complement enzymes except the C3b inactivator belong to the group of serine esterases (Sakai & Stroud, 1974; Ziccardi & Cooper, 1976; Medicus et al., 1976a; Lesavre & Müller-Eberhard, 1978). Physicochemical characteristics of the individual factors are given in table 1.

Two main mechanisms of complement activation are recognized. The classical pathway comprises 9 components, termed C1, C2, C3, C4, C5, C6, C7, C8 and C9. C1 is assembled by three subcomponents: C1q, C1r and C1s held together by Ca^{++} . The alternative pathway includes the components C3, factor B, factor D and properdin and enters the classical pathway at the C3 level. Both pathways include the components C5 to C9.

Activation of the complement system is brought about by a sequential reaction, in which complexes between components are formed and highly specific proteolytic enzymes are generated. The activation is regulated by spontaneous decay of activated components and by the action of specific control proteins. Hitherto, the C1 inactivator, the C3b inactivator, B1H, the C4 binding protein and the anaphylatoxin inactivator have been defined. Recently, the S-protein was discovered and found to be an inhibitor of the C5b-6 complex.

Nomenclature. The nomenclature used is in accordance with conventions resulting from discussions at several international conferences (Austen et al., 1970; Austen et al., 1974). Thus, the abbreviation C stands for complement. The components of the classical pathway are denoted numerically and capital letters are used for the components of the alternative pathway. P stands for properdin, B stands for factor B, which is synonymous with the C3 proactivator (C3 PA) and glycin-rich β glycoprotein (GBG). D denotes factor D, earlier known as precursor of C3PAase or pro-GBGase. Fragments of complement

Table 1.

Physicochemical characteristics of proteins of the complement system

Component	Approximate molecular weight $\times 10^3$	Electro- phoretic mobility	Serum con- centration (mg/l)	Fragment
C1q	390	γ_2	190	
C1r	83	β	*	
C1s	83	α_2	120	
C4	209	β_1	430	C4a, C4b, C4c, C4d
C2	117	β_2	30	C2a, C2b
C3	190	β_1	1300	C3a, C3b, C3c, C3d, C3e
C5	206	β_1	75	C5a, C5b
C6	95	β_2	60	
C7	120	β_2	60	
C8	160	γ_1	80	
C9	80	α	160	
Properdin	220	γ_2	25	
Factor B	100	β_2	240	Ba, Bb
Factor D	23	α	*	
C1 inactivator	105	α_2	180	
C3b inactivator	93	β_2	50	
B1H	150	β_1	520	
C4 binding protein	(540)	β_2	*	
Anaphylatoxin inactivator	325	α	*	

* Not known

From: Austen, 1979

components arising on cleavage or activation are denoted by addition of a small letter to the symbol representing the complement component in question, e.g. C3a, C3b, C4a and C4b. Enzyme activity residing in a component or complex of components is indicated by a bar over the component number, e.g. $\overline{\text{C1s}}$, $\overline{\text{C42}}$. For complexes the fragment of each component can be given, e.g. $\overline{\text{C4b2a}}$, $\overline{\text{C3b8b}}$. The $\overline{\text{C1}}$ inactivator is abbreviated to $\overline{\text{C1}}$ IA, synonyms are $\overline{\text{C1}}$ esterase inhibitor and $\overline{\text{C1}}$ inhibitor. The C3b inactivator is shortened to C3b INA and is identical with the conglutinin activating factor (KAF). AI stands for the anaphylatoxin inactivator. C3 NeF denotes the C3 nephritic factor, and C4 BP stands for the C4 binding protein.

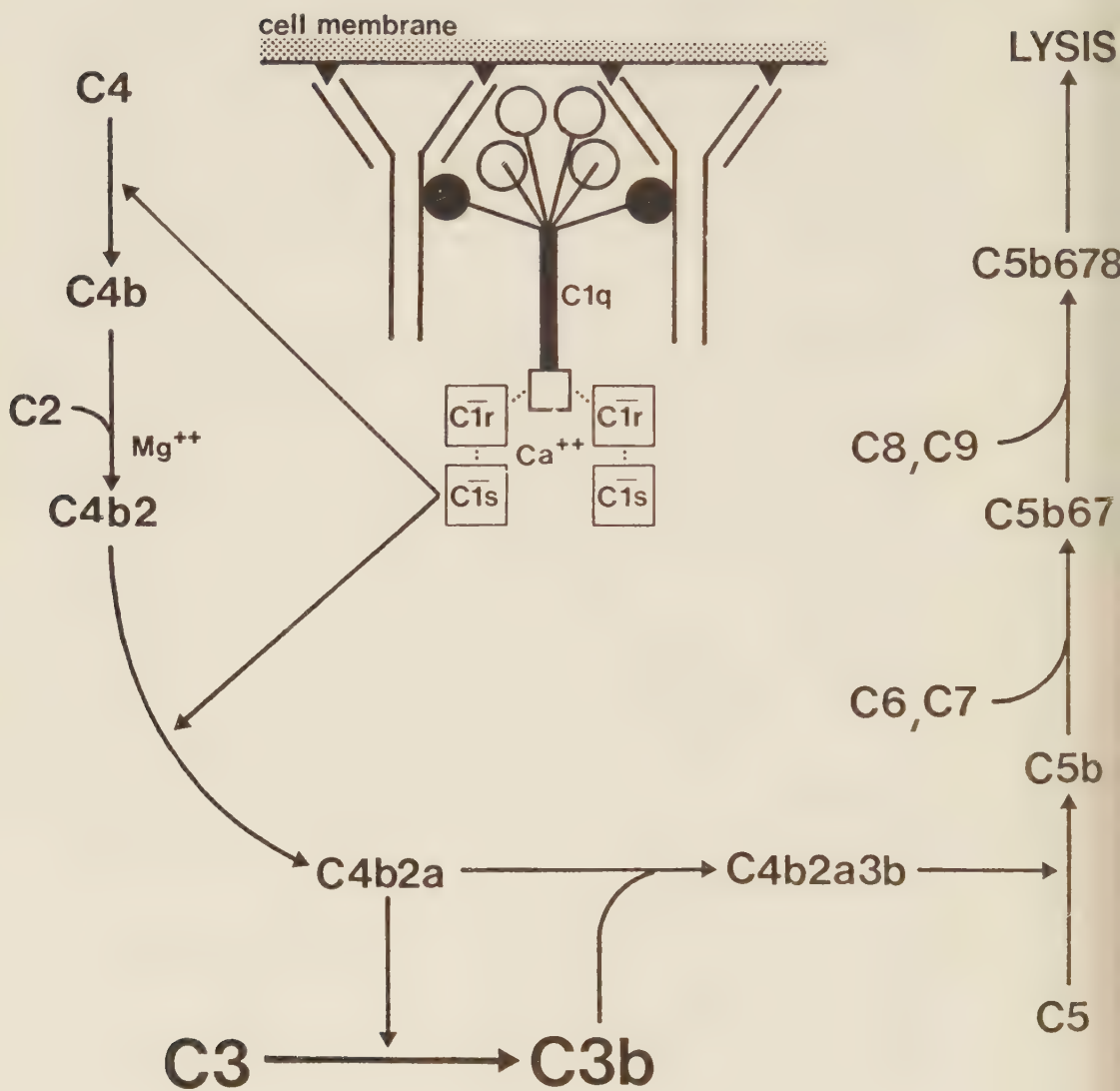
The classical pathway. (Cf Müller-Eberhard, 1975; Porter, 1977; Fearon & Austen, 1978). The first component of the classical pathway, C1, is a calcium-dependent complex of three distinct proteins, called C1q, C1r and C1s (Lepow et al., 1963; Naff et al., 1964). The biologically most important activators of C1 are immune complexes containing IgM or IgG immunoglobulins (Augener et al., 1971).

C1q consists of six non-covalently linked subunits composed of globular and collagen-like regions (Reid et al., 1972; Yonemasu & Stroud, 1972). Ultrastructural studies of C1q by electron microscopy have revealed six peripheral globular portions joined by strands to a central fibril-like structure (Svehag et al., 1972; Shelton et al., 1972). The valency of C1q for binding to immunoglobulins is six (Müller-Eberhard & Calcott, 1966; Schumacher et al., 1976). The globular portions of the C1q molecule correspond to these binding sites (Knobel et al., 1974; Reid & Porter, 1975).

Activation of C1q requires Ca^{++} and the interaction with at least two IgG binding sites closely situated (Müller-Eberhard & Calcott, 1966; Borsos, 1971). Binding and activation of C1q results in a proteolytic cleavage of C1r leading to expression of an enzymatic activity (Ziccardi & Cooper, 1976). The only known natural substrate of $\overline{\text{C1r}}$ is the proenzyme C1s, which on action of $\overline{\text{C1r}}$ is converted to $\overline{\text{C1s}}$ by cleavage of a single peptide bond (Sakai & Stroud, 1974; Valet & Cooper, 1974a,b). Both $\overline{\text{C1r}}$ and $\overline{\text{C1s}}$ are inhibited by the $\overline{\text{C1}}$ inactivator (Gigli et al., 1968; Harpel & Cooper, 1975).

The immunoglobulins IgG1, IgG2, IgG3 and IgM, complexed to an antigen or physically aggregated, bind and activate C1. The binding

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site for C1q on IgG is situated in the C_H2 domain of the Fc region (Ellerson et al., 1972), while that on IgM is found in the C_H4 domain (Hurst et al., 1974).

C1 can also be activated by means other than immune complexes. Thus, C1 is activated by complexes of C-reactive protein (CRP) with phosphorylcholine-containing substances (Volanakis & Kaplan, 1971; Kaplan & Volanakis, 1974). Similarly, C1 is activated by complexes of CRP with a variety of polycations (Siegel et al., 1974; Siegel et al., 1975) and by interactions of polyanions and polycations (Rent et al., 1975). In this way the complement system may interfere with inflammatory reactions associated with the production of acute phase reactants.

C1s cleaves C4 into two fragments termed C4a and C4b (Patrick et al., 1970; Budzko & Müller-Eberhard, 1970). Some of the C4b molecules bind to cell membranes or immune complexes by a labile binding site (Law et al., 1980). The next substrate of C1s is C2 which, after Mg⁺⁺ dependent complex formation with C4b, is cleaved into C2a and C2b (Gigli & Austen, 1969). The complex formed, C42 (C4b2a), has enzymatic activity, the classical C3 convertase. The C3 cleaving enzyme C42 resides in the C2 portion of the complex (Polley & Müller-Eberhard, 1968). During the cleavage of C2 a fragment with kinin-like activity is released into the fluid phase (Donaldson et al., 1970; Lepow, 1971). The capacity of C4b to bind C2 is destroyed by the C3b inactivator, which in the presence of C4 binding protein cleaves the molecule (Cooper, 1975; Fujita et al., 1978; Scharfstein et al., 1978; Gigli et al., 1979; Fujita & Nussenzweig, 1979). The final fragmentation products of C4b are C4c and C4d (Shiraishi & Stroud, 1975).

C42, the classical C3 convertase, splits C3 into C3a and C3b (Müller-Eberhard et al., 1967; Bokisch et al., 1969). The latter binds to cell membranes and immune complexes by a labile site on the molecule (Bokisch et al., 1975; Capel et al., 1978). The interaction is probably an ester linkage between the labile binding site of the C3b and the receptive surfaces (Law et al., 1979).

Together with C42, C3b generates C423b (C4b2a3b), which cleaves C5 into the fragments C5a and C5b (Cooper & Müller-Eberhard, 1970; Nilsson et al., 1975). C5b and C6 fuse to form a bimolecular complex (Lachmann & Thompson, 1970; Podack et al., 1978a), which after col-

lision with C7 gives rise to the trimolecular $\overline{C5b67}$ complex. $\overline{C5b67}$ transiently expresses a membrane binding site (Podack et al., 1978b; Podack & Müller-Eberhard, 1978). During the final steps C8 and C9 bind and the complex $\overline{C5b6789}$ acquires membranolytic activity (Manni & Müller-Eberhard, 1969; Kolb & Müller-Eberhard, 1974). If the S-protein is bound to the forming C5b-9 complex, the complex cannot attach to surfaces of cells and the lytic reaction is inhibited (Podack et al., 1978a; Podack & Müller-Eberhard, 1979). Interaction of the C5b-9 complex with the cell membrane causes changes in the lipid bilayer structure of the membrane leading to disturbances of the normal osmotic barrier of the cell (Bhakdi & Tranum-Jensen, 1978; Podack & Müller-Eberhard, 1978).

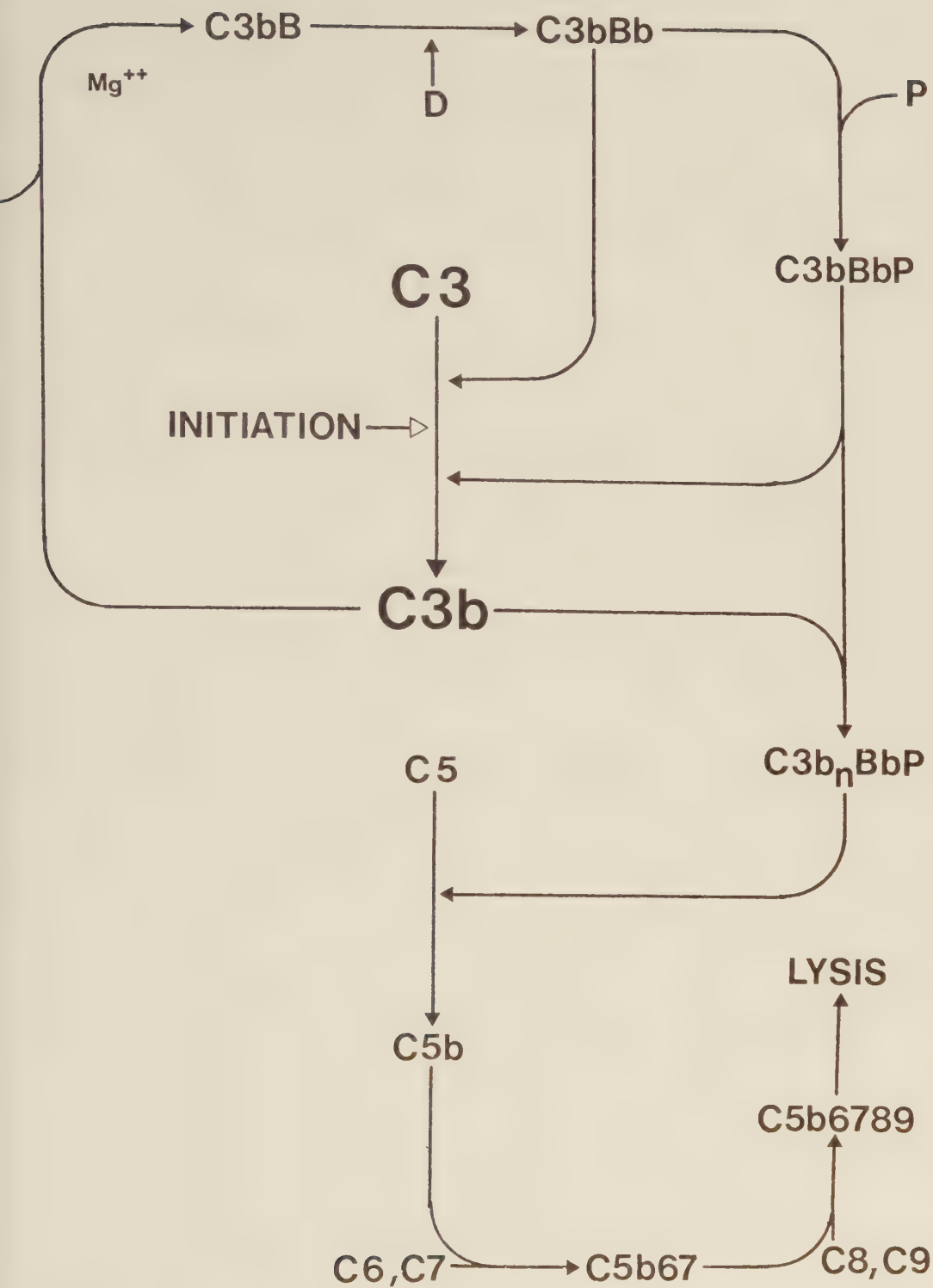
In the presence of C7, C8 and C9 fluid phase $\overline{C5b6}$ complexes can bind to membranes of cells not sensitized with specific antibodies and thereby cause destruction of "innocent bystander" cells. The phenomenon is called reactive lysis (Thompson & Lachmann, 1970; Lachmann & Thompson, 1970; Goldman et al., 1972).

The alternative pathway. (Cf Götze & Müller-Eberhard, 1976; Fearon & Austen, 1978; Fearon & Austen, 1980.) The alternative pathway was originally described by Pillemer and coworkers (1954, 1956). They claimed that serum factors distinct from C1, C2 and C4 interacted with zymosan at 37°C with the formation of a zymosan-protein complex capable of activating C3. Properdin was an essential component in the activation mechanism; hence the alternative pathway is also referred to as the properdin system.

It is now known that the pathway comprises properdin, C3b (in older literature termed factor A), factor B and factor D. Activation of the pathway is not dependent of antibodies and is triggered off by a variety of substances, including microbial polysaccharides (Pillemer et al., 1955), lipopolysaccharides from Gram-negative bacteria (Pillemer et al., 1955; Gewurz et al., 1968; Marcus et al., 1971) and teichoic acid from pneumococci (Winkelstein & Tomasz, 1978). The alternative pathway thus provides the non-immune individual with a type of antimicrobial defence operating until specific antibodies are formed.

The initial events in the activation mechanism have been the subject of debate since the rediscovery of the properdin system. One of

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the essential questions concerns the initial formation of C3b, a cleavage product of C3 necessary for the formation of the C3 and C5 converting enzymes of the pathway. Lachmann & Halbwachs (1975) proposed the "C3b-tick over" hypothesis according to which C3b is continuously being formed in plasma by some mechanism.

An essential part of the alternative pathway is the positive feedback mechanism of C3 activation, called amplification loop (Müller-Eberhard & Götze, 1972; Nicol & Lachmann, 1973; Fearon et al., 1973) and in which $\overline{C3bBb}$ by cleaving C3 recruits more C3b for its own formation.

Generation of the alternative pathway convertase requires C3b, factor B, factor D and Mg^{++} (Müller-Eberhard & Götze, 1972). In the presence of Mg^{++} factor B is complexed to C3b, after which factor B is sensitive to cleavage by factor D (Vogt et al., 1975; Vogt et al., 1977; Lesavre & Müller-Eberhard, 1978). It has been claimed that uncleaved factor B in complex with C3b exerts a limited C3 converting activity (Day et al., 1976; Daha et al., 1976a). The observations have, however, been questioned (Lesavre & Müller-Eberhard, 1978). The C3 cleaving efficiency is greatly enhanced by cleavage of factor B in the C3bB complex with factor D (Vogt et al., 1975). The C3 cleaving activity is located in the Bb molecule (Medicus et al., 1976a). The $\overline{C3bBb}$ complex is labile owing to dissociation of Bb, which appears in the fluid phase in an inactive form, Bi (Fearon et al., 1973). The half-life of the complex is significantly increased by the binding of properdin to the C3b molecule (Schreiber et al., 1975; Fearon & Austen, 1975). Properdin retards the spontaneous decay of $\overline{C3bBb}$ (Fearon & Austen, 1975). Properdin also protects C3b from cleavage by C3b INA (Medicus et al., 1976b).

The formation and function of $\overline{C3bBb}$ is controlled by B1H, which competes with factor B for association with C3b (Conrad et al., 1978). Binding of B1H to C3b blocks the formation of C3bB, dissociates factor B that is bound to C3b and increases the susceptibility of C3b to cleavage by C3b INA (Whaley & Ruddy, 1976; Weiler et al., 1976).

It was recently shown that some cells capable of activating the alternative pathway contain small amounts of sialic acid (Fearon, 1978; Pangburn & Müller-Eberhard, 1978; Kazatchkine et al., 1979b). Depending on the surface structure alternative pathway activators have the ability to protect bound C3b from being complexed with B1H and thereby

allow assembly of the C3 converting enzyme $\overline{C3bBb}$.

Factor D occurs in plasma as an active enzyme not inhibited by the normal plasma protease inhibitors. It has restricted specificity and cleaves factor B only after substrate modulation by binding to C3b (Vogt et al., 1975; Lesavre & Müller-Eberhard, 1978).

Adjacent C3b molecules extend the action of $\overline{C3bBb}$. A new enzyme is formed, $C3b_nBb$, which besides being a C3 convertase, also cleaves C5 into C5a and C5b, thus entering the C5 to C9 sequence of the classical pathway (Medicus et al., 1976c; Daha et al., 1976b; von Zabern et al., 1979).

In membranoproliferative glomerulonephritis an IgG class auto-antibody specific for the C3 convertase of the alternative pathway is found (Daha et al., 1976c; Davis et al., 1977; Scott et al., 1978; Daha et al., 1978; Schreiber & Müller-Eberhard, 1978). The antibody is called C3 nephritic factor (C3 NeF) and binds to the $\overline{C3bBb}$ complex which is stabilized resulting in enhanced activity. This is not only due to retardation of the spontaneous decay (Schreiber et al., 1975; Daha et al., 1976c) but also to decreased susceptibility of the complex to the action of β_1H and the subsequent cleavage by C3b INA (Weiler et al., 1976). When localised sufficiently close to each other, C3 NeF molecules bind and activate C1q in the classical pathway (Sobel et al., 1979; Daha et al., 1979).

Control mechanisms of complement. Activated $C1r$ and $C1s$ are irreversibly inhibited by binding to $C1\ IA$ (Gigli et al., 1968; Harpel & Cooper, 1975). $C1\ IA$ also inhibits other proteases not related to the complement system, such as plasma kallikrein, plasmin and activated Hageman factor (Ratnoff et al., 1969; Forbes et al., 1970).

In cooperation with the C4 binding protein, C3b INA cleaves C4b, thus preventing binding of C2 (Cooper, 1975; Scharfstein et al., 1978; Fujita et al., 1978; Fujita & Nussenzweig, 1979; Gigli et al., 1979).

C3b is cleaved by C3b INA and the cleavage is facilitated after binding of C3b to β_1H (Ruddy & Austen, 1971; Gitlin et al., 1975; Weiler et al., 1976; Whaley & Ruddy, 1976; Pangburn et al., 1977; Whaley & Thompson, 1978), thus preventing binding of factor B. The Bb fragment in $\overline{C3bBb}$ is displaced by β_1H , also when the complex is stabilized by properdin (Weiler et al., 1976).

The activity of the C3 convertases $\overline{C42}$ and $\overline{C3bBb}$ is limited by spontaneous decay and by labile binding sites on C4b and C3b which

precludes excessive destruction of "bystander cells" by immune lysis.

The anaphylatoxins C3a and C5a, which are generated on activation of C3 and C5, respectively, are controlled by the anaphylatoxin inactivator which has carboxypeptidase B activity, and removes the C-terminal amino acid arginine from C3a and C5a. The spasmogenic activity of the molecules is destroyed but C5a retains the chemotactic activity (Bokisch & Müller-Eberhard, 1970).

The C5b-9 complex is inhibited by the S-protein, which on binding to the complex makes attachment to the cell membrane impossible (Podack et al., 1978; Podack & Müller-Eberhard, 1979).

Biological activities of complement. The complement system plays an essential role in the defence of the host against immune complexes, bacteria and certain viruses. Complement, through its potent fragmentation products, is also an important mediator of the inflammatory reaction.

Deposition of the C5b-9 complex on cell membranes by activation of the classical and alternative pathways leads to immune cytotoxicity.

Immune adherence is the binding of particles or immune complexes carrying C3b on their surfaces to different cells equipped with C3b receptors (immune adherence receptors). Such cells are human erythrocytes, polymorphonuclear leukocytes, macrophages and B-lymphocytes. Immune adherence receptors have also been shown on renal glomerular cells (Gelfand et al., 1975). A detailed review has been given by Bianco and Nussenzweig (1977). Immune adherence may also be mediated by C4b, but is less efficient than by C3b (Cooper, 1969).

Immune adherence to phagocytes is the basis of opsonization in the presence of complement and may trigger off release of lysosomal enzymes.

A function that may play an important role in immune complex diseases is the solubilisation of immune complexes in fresh serum, which is mainly a function of activation of the alternative pathway (Takahashi et al., 1976).

Complement mediated virus neutralisation has been observed in some systems (Cooper et al., 1976; Leddy et al., 1977; Mills & Cooper, 1978; Mills et al., 1979).

Activation of C3 and C5 leads to generation of the small peptides C3a and C5a, which are called anaphylatoxins (cf Hugli & Müller-Eberhard, 1978). C3a and C5a release vasoactive amines from mast cells

and basophilic leukocytes. C5a has a chemotactic effect on polymorphonuclear leukocytes (Chenoweth & Hugli, 1978). Recently, the C4a fragment of C4 was shown to possess anaphylatoxin activity. C4a expresses about 1 per cent of the spasmogenic activity of C3a (Gorski et al., 1979).

C3e, an acidic fragment of C3, increases the number of circulating leukocytes by mobilization of the cells from the bone marrow (Rother, 1972; Ghebrehiwet & Müller-Eberhard, 1979). The physiological significance of C3e has not yet been established.

Evidence from several investigations points towards a role of complement in the antibody response (Ellman et al., 1970; Pepys, 1976; Ochs et al., 1978). Complement cleavage products mediate the release of lymphokine from B-lymphocytes in vitro (Wahl et al., 1974; Koopman et al., 1976). Complement also induces and regulates macrophage motility (Götze et al., 1977a; Bianco et al., 1979).

Inborn errors of complement components. (Cf Agnello, 1978.) Genetically determined deficiencies have been described for many complement components. Some of them are associated with disease, and clearly implicate the protective functions of the complement. The deficiencies are rare and inherited as autosomal recessive traits.

Deficiencies of the classical components C1r, C1s, C2 and C4 are associated mainly with the clinical manifestation of immune complex diseases. Deficiency of C3 enhances the susceptibility to bacterial infections, as does deficiency of the C3b inactivator with a state of C3 hypercatabolism as a result (Abramson et al., 1971; Alper et al., 1972). Deficiencies of the components C5 to C9 are often associated with disseminated infections with *Neisseria*-species.

Hereditary angio-edema (HAE) is due to a partial deficiency of the C1 inactivator, and secondary deficiencies of C4 and C2 develop with SLE-like syndromes as a possible consequence.

Deficiency of properdin has recently been described in a family with cases of lethal meningococcal infections (Braconier et al., 1981).

THE PRESENT INVESTIGATION

The object of the thesis was to elucidate different activation mechanisms of the complement system.

The first part deals with the classical pathway. The complement profile in acute and recurrent pneumococcal otitis media was analysed. Partly extended from this investigation was the study of C1 subcomponent complexes and C1 activation, which included the development of a simple method for the purification of C-reactive protein. The influence of C1 subcomponents on the positive feedback mechanism of C3 cleavage was investigated, providing a link between classical and alternative pathway activation.

The second part concerns the alternative pathway. Complement activation by this mechanism was demonstrated in patients with adverse reaction to dextran (Macrodex^R) and in meningococcal septicemia. The possibility that activated properdin is capable of cleaving C3 and thus initiates the alternative pathway was also examined.

The third part of the thesis elucidates the effects of granulocyte proteases on the complement components C3 and C5.

GENERAL METHODOLOGY

Samples for analyses of complement components were collected as serum and EDTA-plasma (EDTA, 10 mmol/l) and were prepared frozen in aliquots within 4 hours of withdrawal at -80°C . The individual components were quantified with the electroimmunoassay (Laurell, 1972).

The components C4, C3, factor B, C3b INA and C5 were quantified in EDTA-plasma. Dilution before electrophoresis was done with barbital buffer, 75 mmol/l, containing EDTA, 2 mmol/l, pH 8.6. The electrophoresis was carried out in barbital buffer without Ca^{++} at pH 8.6, 20 V/cm for 4 hours. The concentration of agarose (Miles-Seravac, Maidenhead, England) was 0.6 % w/v.

Properdin was quantified in serum. The electrophoresis was run for 20 h at 4 v/cm in barbital buffer, 75 mmol/l, containing calcium lactate 2 mmol/l, pH 8.6. Agarose (L'Industrie Biologique Française, S.A., Gennevilliers, France) at 1 % w/v was suitable under the conditions used.

For measurement of C1q, phosphate buffer, 0.1 mole/l, pH 6.0 was used. C1r and C1s were determined in serum. For dilution immediately before analysis barbital buffer, 75 mmol/l, pH 8.6, containing EDTA, 2 mmol/l, was used. Electrophoresis was run at 4 V/cm for 18 hours.

The results of the quantification are given as percentages of a serum or an EDTA-plasma reference pool made from 100 apparently healthy blood donors and was said to contain 100 per cent of the component investigated. The normal ranges and the precision of the determinations are those given by Sjöholm (1975) and by Laurell et al. (1978a).

Fragmentation of C3 was detected with the use of crossed immuno-electrophoresis (Laurell, 1965; Ganrot, 1972). EDTA-plasma was strictly used for the analysis. Conversion of factor B was studied in EDTA-plasma with the aid of immunoelectrophoresis (Scheidegger, 1955).

Complexes of C1 subcomponents were studied with crossed immuno-electrophoresis with the first separation run in buffer, pH 8.6, containing calcium lactate 2 mmol/l. The second step was done with EDTA or with Ca^{++} in the buffer and with specific antisera against the C1 subcomponents in the agarose gel, alone or in combination (Laurell et al., 1978b).

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(I, II, III, IV)

Complement profile in acute pneumococcal otitis media (I)

Complement components were studied in 6 children with their first attack of acute pneumococcal otitis media and in 14 children with recurrence. The causative agent was Streptococcus pneumoniae. The strains were isolated from middle ear fluid except in a few patients, in whom specimens were obtained from the nasopharynx. The serotypes and their frequency corresponded to that reported in earlier investigations (Kamme et al., 1970). In recurrent otitis media the second episode was always caused by the same serotype as in the first.

Blood samples were obtained on admission and again 2-3 weeks later, in some cases a third sample was collected 6 weeks after onset.

Specific capsular pneumococcal antibodies were determined by immunofluorescence. No significant differences were found between primary attacks and recurrences. In some patients a significant increase in titre was recorded between the first and second sample. The serum IgG, IgA and IgM levels were invariably within the normal range.

The primary attacks did not differ from recurrences regarding the levels of C4, C3, C5 and factor B. Conversion of factor B was not found in any of the samples. The properdin levels were slightly lower, though not significantly, in the group with recurrences.

The C1 subcomponents C1q, C1r and C1s showed abnormalities. During the acute phase 9 out of 14 of the patients with recurrence and one of the 6 primary cases had a low C1q level combined with high levels of C1r and C1s. Crossed immunoelectrophoresis revealed increased amounts of C1r-C1s-C1 IA complexes and particularly of C1r-C1s complexes containing proenzyme C1r and C1s.

Purification of C-reactive protein (II)

It is known that Ca^{++} strongly influences the charge of C-reactive protein (CRP) (Gotschlich & Edelman, 1967; Ganrot & Kindmark, 1969). If Ca^{++} is present during chromatography on DEAE-cellulose, CRP is weakly bound to the cellulose. Removal of Ca^{++} by chelation with EDTA caused a firm bond and the protein was eluted at a buffer conductance of more than twice that required in the presence of Ca^{++} .

Serum or ascitic fluid from patients with high levels of CRP served as starting material and was applied to DEAE-cellulose in the presence of Ca^{++} . The column was washed with a Ca^{++} -containing buffer at a conductance allowing about 80 per cent of the total protein, including CRP, to pass through in the void volume. After addition of EDTA the void was applied to a second DEAE-column previously equilibrated with a buffer containing EDTA. Most of the protein again appeared in the void volume while CRP was retained on the column. After extensive washing, CRP was eluted by increasing the conductance of the buffer.

Isolated CRP was analysed with immunoelectrophoresis against anti-whole human serum in a concentration of 3 g/l and no contaminating proteins were detected. Electrophoresis on agarose gel gave only one band.

Complex formation between C1 subcomponents and the C1 inactivator on activation of C1 (III)

In vitro complex formation of C1 subcomponents occurring on activation of C1 was studied with crossed immunoelectrophoresis. C1 in serum was activated with proteolytic enzymes, heat aggregated IgG, immune complexes and with complexes of CRP with protamine sulphate.

A. Incubation of normal serum with purified C1r and purified C1s resulted in a decrease of C1r and C1s associated with macromolecular C1. Using varying antisera against C1r, C1s and C1 IA, crossed immunoelectrophoresis revealed C1s in an α_2 position in complex with C1r and C1 IA (C1r-C1s-C1 IA). C1q had shifted to a more cathodal electrophoretic position, which corresponded to that of isolated C1q. Incubation with trypsin gave the same result. Plasmin, even when used in high concentrations (7.5 CTA units per ml), did not influence the electrophoretic C1s pattern in serum, and it was concluded that plasmin does not activate C1 in normal serum.

B. Heat aggregated IgG increased the amount of C1r-C1s-C1 IA complexes. C1q could not be detected with crossed immunoelectrophoresis, probably due to binding to the IgG aggregates.

C. Immune complexes, either as ovalbumin-anti-ovalbumin complexes or as cell-bound antibodies, were incubated with normal serum. Increased amounts C1r-C1s-C1 IA were seen on mixing serum with ovalbumin and anti-ovalbumin over a wide range of ovalbumin concentrations. The largest amounts were recorded at equivalence and in antigenic ex-

cess. The use of cell-bound antibodies gave results corresponding to those obtained with heat aggregated IgG.

D. When CRP was complexed with protamine sulphate and added to serum all C1q, C1r and C1s disappeared from the application site on crossed immunoelectrophoresis. The C1s protein was again found in the α_2 position and the mobility of C1q had shifted towards the cathode.

E. Two pathological sera containing C1r-C1s complexes were studied: one containing normal amounts of C1q, the other C1q at a level less than 1 per cent of the normal. Following incubation with C1r and C1s the serum containing normal C1q showed a decrease of the macromolecular C1. The C1r-C1s complex disappeared in both sera on incubation with C1r or C1s, and increased amounts of C1r-C1s-C1 IA complexes were found. Incubation of the serum containing C1q with aggregated IgG gave rise to the same electrophoretic pattern as when the serum was incubated with C1r. In contrast, incubation of the C1q deficient serum with aggregated IgG did not influence the C1s protein pattern. Thus, treatment of serum with aggregated IgG resulted in the disappearance of the C1r-C1s complexes only when macromolecular C1 was available.

F. Normal serum, which contained small amounts of C1r-C1s and C1r-C1s-C1 IA complexes, was filtrated on Sepharose-CL 6B. Two main protein peaks appeared, and all C1q, C1r and C1s, as determined with electroimmunoassay, emerged between the two peaks. Crossed immunoelectrophoresis of the concentrated fractions containing C1q, C1r and C1s showed the same C1s protein pattern as serum before gel filtration. The results support the suggestion that the C1r-C1s and C1r-C1s-C1 IA complexes were preformed in serum.

Normal serum was treated with aggregated IgG at 1 mg/ml. After treatment, almost all C1s was incorporated in C1r-C1s-C1 IA complexes. Subsequent fractionation was carried out using the same column as for untreated normal serum. C1r-C1s-C1 IA complexes in these fractions were identified by using combinations of anti-C1s, anti-C1r and anti-C1 IA in the electroimmunoassay. Analysis with crossed immunoelectrophoresis of the pooled and concentrated fractions confirmed the findings obtained by analysis of pathological sera (Laurell et al., 1976) and established the presence of C1r, C1s and C1 IA protein in α_2 complexes and that the C1r in the complex could be detected by combination of

anti-C1r with anti-C1s.

Initiation of the alternative pathway feedback mechanism by activation of the classical pathway (IV)

To investigate the influence of activation of the classical pathway on the positive feedback mechanism of the alternative pathway, normal serum and C4 deficient serum were incubated with C1r, C1s, C1r-C1s complexes and with activated properdin.

Activation of the feedback mechanism of the alternative pathway was detected by analysing C3 and factor B conversion with crossed immunoelectrophoresis and immunoelectrophoresis, respectively.

Effects of C1s. When C1s was added to normal serum a partial conversion of C3 was noted and factor B was cleaved to Bb. The presence of EDTA prevented cleavage of C3 and factor B. Neither did C1s give rise to cleavage of C3 or factor B in a C4 deficient serum indicating that the observed C3 and factor B conversion in the normal serum appeared as a consequence of classical pathway activation. Treatment of the C4 deficient serum with inulin gave a strong conversion of both C3 and factor B, which showed that the alternative pathway was intact.

Effects of C1r. Addition of purified C1r to normal serum did not cause conversion of C3 or factor B. However, macromolecular C1 was lowered and the amount C1r-C1s-C1 IA complexes was increased, which indicated activation of the classical pathway (Laurell et al., 1976). On further incubation of the C1r treated serum with C1s, C3 was partially converted, while factor B remained uncleaved. This contrasts with the experiment where C1s alone was added to serum. On incubation of the C1r and C1s treated serum with inulin both C3 and factor B were strongly converted.

Effects of C1r-C1s complexes. Incubation of normal serum with C1r-C1s complexes caused conversion of C3 and factor B comparable to what was found in the experiments in which serum was incubated with C1s alone. The amount C1s was the same in both experiments.

Effects of properdin. Normal serum was depleted of properdin by immune adsorption in the presence of EDTA. After adsorption Mg^{++} and Ca^{++} were added to the serum, each at the concentration of 2 mmoles/l. C1s was added to normal and to properdin-depleted serum,

and the C3 and factor B conversion did not differ between the two sera.

When the properdin-depleted serum was treated with inulin both C3 and factor B remained in their native states. However, addition of purified activated properdin to the properdin-depleted serum resulted in fragmentation of the two proteins without any other activating substance present. These findings suggest impairment of the activation of the alternative pathway without affecting the feedback mechanism in the properdin-depleted serum, which could still be activated by the classical pathway C3 convertase on addition of C1s, and by activated properdin.

DISCUSSION

The complex formation between the C1 subcomponents was investigated with the use of crossed immunoelectrophoresis (Laurell, 1965; Ganrot, 1972). The advantage of the method is that it allows detection of protein complexes otherwise difficult to reveal. Furthermore, the composition of the complexes can be studied with the use of various combinations of specific antisera in the gel.

On activation C3 is converted to C3b, which in serum is further degraded to C3c and C3d (Ruddy & Austen, 1971). The electrophoretic mobility of C3b differs very little from that of C3c, but the fragments are clearly distinguished with crossed immunoelectrophoresis.

In serum C1 is a macromolecule composed of C1q, C1r and C1s held together by Ca^{++} (Lepow et al., 1963; Naff et al., 1964). Crossed immunoelectrophoresis of normal serum with anti-C1s in the secondary gel reveals C1s protein at the application site in complex with C1q and C1r and in α_2 position representing a complex of C1r, C1s and C1 IA (Laurell et al., 1976; Laurell et al., 1978b; III). A third species of C1s containing complexes was detected in β_1 position in sera from patients with chronic urticaria and angioedema (Laurell et al., 1977). The β_1 positioned C1s was found to be in a Ca^{++} dependent complex with C1r and the two subcomponents were found to be in proenzyme form (Laurell et al., 1976; Sjöholm, 1979).

Such proenzyme C1r-C1s complexes were found with increased frequency and in high concentrations in patients with a recurrence of otitis media caused by pneumococci (I). Together with lowered serum

levels of C1q and increased amounts of C1s in α_2 position, this was the only significant difference in the immunological parameters observed between patients with and without a recurrence of the otitis. Thus, the levels of C4, C3, C5 and factor B were within the normal range and did not differ between the two groups. This is in accord with later investigations, in which pneumococci were added to normal serum (Prellner, 1979).

It has been shown that incubation of normal serum with pneumococci of different serotypes causes conversion of C3 and that the C3 cleavage is mainly a function of the alternative pathway (Prellner, 1979).

In recurrences in our investigation the levels of properdin were slightly lower than in the primary cases. Prellner (1979) showed a marked fall in the properdin levels on in vitro incubation of normal serum with pneumococci. This was noted also in C2 deficient serum, which reflects involvement of the alternative pathway.

Since all children showed type specific pneumococcal antibodies, activation of the classical pathway was expected. A sensitive parameter of activation of the pathway is the occurrence of increased amounts C1r-C1s-C1 IA complexes (Laurell et al., 1976). If such an increase be accepted as a criterion, 50 per cent of the primary and 69 per cent of the recurrences showed evidence of classical pathway activation. Although alternative pathway activation in vitro is shown to occur on incubation of normal serum with pneumococci (Fine, 1975; Winkelstein & Tomasz, 1977; Prellner, 1979), no fragmentation of factor B was observed in our investigation with the method used. However, complement activation by the alternative pathway, most probably rather limited to the region of the middle ear, might be masked in the circulation by dilution or elimination of the fragments and complexes formed during activation.

The mechanism responsible for the generation of C1r-C1s complexes is not clear. Studies of the C1 subcomponents in disease have shown that the levels of C1q vary more independently than those of C1r and C1s but offer no definite explanation (Pickering et al., 1970; Stroud et al., 1970; Day et al., 1972; deBracco & Manni, 1974; deBracco et al., 1974; Laurell et al., 1977; I). A dynamic dissociation equilibrium of the C1 subcomponents has been reported (Bartholomew & Esser, 1977). It is conceivable that C1q is bound to structures on the pneumococci

at the site of the infection of in the circulation and that the C1r-C1s complexes represent an excess of these factors. A recent study (McKay et al., 1981) describes the generation of C1r-C1s complexes on chromatography of normal serum on Heparin-Sepharose^R in the presence of Ca⁺⁺. C1q remained bound to the heparin, whereas about 80 per cent of the serum content of C1r and C1s was eluted complexed with each other and in proenzyme form. A similar mechanism may explain the appearance of C1r-C1s complexes in primary attacks and recurrences of otitis media caused by pneumococci by binding of C1q to the bacteria without activation of C1s and C1r. This possibility is further supported by the finding of Prellner (1980) that pneumococci, especially the serotypes frequently found in this disease, are capable of binding purified C1q when tested in vitro.

It is well documented that C3 and its fragments play an essential role in the opsonization of pneumococci (Shin et al., 1969; Johnston et al., 1969; Winkelstein et al., 1975). The presence of C1r-C1s complexes in vivo might be a sign of dysfunctional classical pathway activation. An intact alternative pathway capable of generating opsonic C3b might not be sufficient and then lead to recurrences in children.

When complexed with pneumococcal polysaccharide (Kaplan & Volanakis, 1974; Claus et al., 1977) or polycations (Siegel et al., 1974; Seigel et al., 1975) CRP activates the classical pathway. In order to study the activation of C1 by complexed CRP a new method for purifying CRP was developed (II). The method includes two chromatographic separation steps on DEAE-cellulose and utilizes the difference in binding of CRP to DEAE in the presence and absence of Ca⁺⁺. The yield varied between 50 and 60 per cent and the isolated protein was pure, as judged from immunoelectrophoresis against anti-whole human serum and from agarose gel electrophoresis with the preparation analyses in a high protein concentration.

Complexes of the C1 subcomponents, C1q, C1r and C1s, were studied in vitro by incubation of normal serum with known activators of the classical pathway (III). On crossed immunoelectrophoresis with Ca⁺⁺ in the first electrophoretic step (see Methodology) activated C1r and C1s appeared in α_2 position complexed to C1 IA (C1r-C1s-C1 IA). C1r in the C1r-C1s-C1 IA complex is antigenically silent, but can be

demonstrated by analysis combining anti-C1r and anti-C1s in the agarose gel (Laurell et al., 1976; III). The antigenic silence of complexed C1r has since been confirmed by the use of other methods (Ziccardi & Cooper, 1978). Further confirmation was given by Sim et al. (1979), who showed that ^{125}I -labelled C1r or C1s added to normal serum or EDTA-plasma appeared in high molecular weight complexes containing C1 IA. Small amounts of C1r-C1s-C1 IA complexes have invariably been found in normal sera; the C1r-C1s complex has been seen in only 1 per cent of investigated sera (Sjöholm, 1979).

The C1 activators used in this investigation (III) did not produce C1r-C1s complexes in vitro. This lends further support to our view that the C1r-C1s complexes formed in vivo partly, as a consequence of C1q binding to various structures appearing in disease, compete with C1r-C1s for C1q.

C1r-C1s complexes present in serum disappeared on incubation with C1r and C1s leading to formation of C1r-C1s-C1 IA. In contrast, aggregated IgG did not transform the C1r-C1s complex to C1r-C1s-C1 IA in C1q-deficient sera (III). On addition of C1q to C1q deficient sera macromolecular C1 (C1qrs) is formed and incubation with aggregated IgG results in the formation of C1r-C1s-C1 IA (Laurell et al., 1976). These observations indicate that the subcomponents in the C1r-C1s are in proenzyme form capable of binding C1q.

CRP complexed to protamine sulphate resulted in dissociation of C1 into C1r-C1s-C1 IA and C1q, as did on incubation with the proteolytic enzymes C1r, C1s and trypsin. Plasmin did not cause any C1 activation in normal serum as revealed by formation of C1r-C1s-C1 IA complexes even when high concentrations of the enzyme were used. It has, however, been reported that purified C1s is activated by plasmin (Ratnoff & Naff, 1967).

Disassembly of C1 in serum into C1r-C1s-C1 IA complexes and C1q detected in a cathodal electrophoretic position corresponding to that of purified C1q, was originally shown by Laurell et al. (1976, 1977). In the present investigation (III) the disassembly was found to be a consequence of C1 activation in serum by various C1 activators. Using purified C1 subcomponents, similar results have been obtained by Ziccardi and Cooper (1979).

May and Frank (1973) reported a new complement mediated cytolytic mechanism in vitro, which they called the C1 bypass activation mechanism. On activation of C1 the complement sequence was triggered off at the C3 level, thus bypassing C4 and C2. In another investigation (Volanakis et al., 1976) it was found that purified C1s induced conversion of factor B and consumption of hemolytic C3 and C5 in sera genetically deficient in C2 or C4. In contrast, our immunochemical studies (IV) with incubation of EDTA-chelated or C4 deficient serum with C1s did not show signs of C3 or factor B conversion. No explanation can be offered for this, which might, however, depend on differences in the analytical techniques used.

When C1r was added to normal serum, macromolecular C1 decreased and the amounts of C1r-C1s-C1 IA complexes increased. C3 and factor B were not fragmented as would be expected from the experiments with C1s. This absence of fragmentation needs further investigation. Also the observation that addition of C1s to C1r treated serum only gave rise to C3 conversion is difficult to explain.

The finding in (IV) shows evidently that $\overline{C4_2}$ formed on activation of the classical pathway initiates the positive feedback mechanism of C3 cleavage of the alternative pathway. Similar results were obtained by Mak et al. (1977), who showed factor B consumption in normal serum on incubation with preformed $\overline{C4_{oxy}2}$ complexes. Parenthetically, this means that factor B conversion cannot be used in the differentiation between classical and alternative pathway activation.

According to the current view of alternative pathway activation, properdin acts only as a stabilizer of the C3/C5 convertase (Fearon & Austen, 1975; Medicus et al., 1976b). Inulin treatment of normal serum activates the alternative pathway. As expected (Pillemer et al., 1955; Götze & Müller-Eberhard, 1974), no conversion of C3 occurred on addition of inulin (IV), when normal serum was depleted of properdin. On addition of C1s to the properdin depleted serum, the C3 and factor B conversion was of the same magnitude as when C1s was added to normal serum. This suggests that properdin is not necessary for the fluid phase function of the classical pathway C3 convertase, $\overline{C4_2}$.

These results again raise the question of the role of properdin

in the alternative pathway. It is still not clear what role activated properdin plays in the initiation of the pathway. Whether various molecular forms of properdin can explain the possible dual functions of properdin as an initiator and a stabilisator of the alternative pathway must abide further research (see V).

THE ALTERNATIVE PATHWAY

(V, VI, VII)

Effects of properdin on C3 (V)

Properdin was purified from normal human serum by euglobulin precipitation, DEAE-cellulose chromatography and Sephadex G 200 filtration. The final product reacted in immunoelectrophoresis with two precipitin arcs when tested against anti-properdin. The more anodal arc was faint and when tested against anti-whole human serum and anti-IgG it was found to contain IgG. The activity of the isolated properdin was tested as outlined by Götze and Müller-Eberhard (1974). Consistent with earlier reports, incubation of the properdin preparation with fresh normal serum resulted in a strong conversion of C3.

C3 was purified according to the method of Nilsson and Müller-Eberhard (1965). The preparation gave only one precipitation arc when tested against anti-whole human serum in a concentration of about 3 mg/ml and failed to react with antisera against factor B, C5 and plasminogen. Incubation of the isolated C3 for 30 minutes at 37°C did not cause any proteolytic cleavage of the molecule demonstrable with crossed immunoelectrophoresis. Reference C3b was prepared by short time trypsination of purified C3 (Cochrane & Müller-Eberhard, 1968).

Isolated properdin and isolated C3 were mixed in the ratio of 1:10 and 1:100 w/w and incubated for 30 minutes at 37°C. Subsequent analysis of C3 with crossed immunoelectrophoresis revealed a fragment with the same electrophoretic mobility as C3b. The degree of C3 conversion was dose-dependent. Soyabean trypsin inhibitor or EDTA did not influence the activity of the properdin preparation. Addition of anti-properdin to the purified properdin before incubation diminished or eliminated the C3 conversion.

Complement profile in dextran induced shock (VI)

The complement components C1q, C4, C3, C5, factor B and C3b INA were quantified in EDTA-plasma obtained from 12 patients who reacted adversely to intravenous infusion of dextran (Macrodex^R). For control, the same analyses were done in 10 patients who received dextran without any adverse reaction.

The values of C1q, C4, factor B, C5 and C3b INA did not differ

significantly between the two groups or between the samples obtained during shock and two hours later.

In five of the reactors C3 was converted and fragmentation of factor B was observed in the EDTA-plasma obtained during shock. In three of these patients the C3 and factor B conversion was still demonstrable in plasma obtained 2 hours later. The serum levels of C3 and factor B were normal in the samples obtained after 1 week. In these five reactors the C3 levels in the 2-hour sample were lower than those in the seven reactors without C3 conversion ($P < 0.05$) and those in the controls ($P < 0.05$).

All plasma samples were screened for the presence of specific dextran antibodies. Double diffusion technique capable of measuring specific antibody down to 40 μg per ml did not reveal any dextran antibodies.

Complement profile in meningococcal septicemia (VII)

The complement components C1q, C4, C3, factor B, C5 and properdin were quantified with electroimmunoassay in EDTA-plasma and in serum from a patient with severe meningococcal septicemia.

In the acute phase of the disease all components tested, except C4 and C5, were depressed. Three days after onset C3 had returned to normal levels, whereas C1q and properdin remained subnormal. On the fifth day, when properdin tended to normalize, C1q was still remarkably low.

Analysis of the C3 component with crossed immunoelectrophoresis during the acute phase showed a substantial conversion of the molecule as well as partial conversion of factor B to Bb. On the third day C3 and factor B were still converted, but to a lesser degree. Five days after onset both C3 and factor B appeared in the native form.

DISCUSSION

Activation of the alternative pathway of the complement system can be brought about by naturally occurring polysaccharides (Pillemer et al., 1955; Burger et al., 1975), endotoxic lipopolysaccharides (Pillemer et al., 1955; Gewurz et al., 1968; Marcus et al., 1971) and rabbit erythrocytes (Platts-Mills & Ishisaka, 1974). Many theories have been proposed to explain the initial events in the

activation of the pathway, including the primary generation of C3b. Lachmann and Halbwachs (1975) suggested a continuous low level generation of C3b by a hitherto not precisized mechanism. Schreiber et al. (1978) have presented a model in which native C3 and factor B normally form a reversible complex in plasma, which is activated by factor D to become a labile initial C3 cleaving enzyme.

It now seems evident that the ability to activate the alternative pathway is related to protection of bound C3b from destruction by the control proteins B1H and C3b INA (Fearon & Austen, 1977; Schreiber et al., 1978; Fearon, 1978). Membrane-associated sialic acid and heparin promotes binding of B1H to cell-bound C3b thereby inhibiting the formation of the C3 convertase $\overline{C3bBb}$ (Fearon, 1978; Pangburn & Müller-Eberhard, 1978; Kazatchkine et al., 1979a,b).

An embarrassing uncertainty exists concerning different types of properdin. It is assumed that properdin in serum is present in a precursor form (Minta, 1976). Native properdin isolated from fresh and untreated serum by a new purification procedure (Götze et al., 1977b), can interact with the assembled, labile C3 convertase, C3bBb, and thereby stabilize the enzyme. It cannot induce formation of C3 convertase in serum, nor can it bind to C3b (Medicus et al., 1980).

Properdin eluted from zymosan is capable to induce consumption of C3 in serum (Pillemer et al., 1954; Pensky et al., 1968), as is properdin prepared from euglobulins of human serum precipitated at low ionic strength and acid pH (Götze & Müller-Eberhard, 1974). Addition of such activated properdin to fresh human serum or to properdin depleted serum results in the activation of the alternative pathway, as judged by changes in the electrophoretic mobilities of C3 and factor B (Götze & Müller-Eberhard, 1974; Minta, 1975; V).

The events leading to conversion of precursor properdin, also called native properdin, to the activated form remains unclear. Minta (1976) found that activation of properdin resulted in reduction in molecular weight, loss of some antigenic determinants and, as described also by others (Spitzer et al., 1976), an alteration in electrophoretic mobility. Chapitis and Lepow (1976)

have described multiple sedimenting species of properdin antigen in serum on ultracentrifugal analyses. They identified C3 as a constituent of at least one of the heavier sedimenting species. Stitzel and Spitzer (1974) isolated a protein from normal human serum, which they called properdin convertase. Immunoelectrophoretic analysis showed that this factor, which was generated on incubation of normal serum with zymosan, caused a cathodal shift of properdin in serum (Stitzel & Spitzer, 1974; Spitzer et al., 1976). This altered properdin cleaved C3 in serum. The same reactivity of properdin was observed when isolated components, properdin convertase, properdin and C3, were mixed and incubated. They also noted that antibody to properdin abolished the C3 cleaving capacity of the activated properdin and that factor B was not necessary for the C3 conversion.

When working with properdin, purified in a native state, from untreated normal serum, Götze et al. (1977b) claimed that the transition of precursor properdin to activated properdin is nonenzymatic. It is thought to be the result of a conformational change of the molecule, since immunoelectrophoresis and polyacrylamide electrophoresis revealed no charge or size differences between precursor and activated properdin. It has also been shown that activated properdin can revert to the native form by the use of methods involving mild protein denaturation and that native and active properdin have identical N- and C-terminal amino acid sequences (Medicus et al., 1980).

Our findings (V) do correlate well with the results of Stitzel and Spitzer (1974, 1976). Our properdin preparation caused C3 conversion when added to fresh normal serum in the absence of an activator of the alternative pathway, indicating that the purified properdin used in the further analyses was in an activated state. In the final step of the isolation procedure the properdin was eluted from the Sephadex G 200 column in the molecular weight range of 200,000 daltons, which is in agreement with the known molecular weight of properdin. The C3 converting activity on purified C3 was independent of divalent cations, was blocked by soyabean trypsin inhibitor and abolished by the addition of anti-properdin. The C3 preparation did not contain C3b, as judged from crossed immunoelectrophoresis, or factor B,

and it was stable on incubation at 37°C, indicating that contamination with any proteolytic enzyme was unlikely. The C3 conversion was dose-dependent and C3b formed was not further degraded on prolonged incubation with the properdin preparation (IV).

Our experimental findings (V) do not lend support to the concept of alternative pathway activation put forward by Schreiber et al. (1978). Further investigations seem necessary to elucidate the background to the initial steps in the activation mechanism, especially concerning the initial formation of C3b. Different molecular species of properdin may have different functions. Thus, activated properdin might play a role as an activator of the alternative pathway besides its stabilizing function of the otherwise labile C3/C5 convertases (Schreiber et al., 1975; Fearon & Austen, 1975; Medicus et al., 1976b).

Complement components were studied in adverse reactions occurring on intravenous administration of dextran (Macrodex^R) and in a case of meningococcal septicemia with shock (VI, VII). Native dextrans and bacterial lipopolysaccharides activate the complement system by the alternative pathway (Pillemer et al., 1955; Gewurz et al., 1968; Marcus et al., 1971; Burger et al., 1975). The degree of complement activation by native dextrans increases with molecular weight starting at MW 60,000 (Hänsch et al., 1979).

Quantification of complement components in sera from patients with adverse reactions to intravenous administration of dextran (Macrodex^R) produced evidence of alternative pathway activation. Normal C1q and C4 values were found. No specific dextran antibodies were detected with the technique used (VI).

In another investigation, depressed levels of C1q and C4 combined with minor reduction of factor B were observed in cases with severe dextran-induced anaphylactoid reactions. In these cases specific dextran-antibodies were found by the use of hemagglutination (Hedin, 1977). As in our investigation (VI) conversion of both C3 and factor B was noted.

The reason for the discrepancy between the two studies is not clear. It is conceivable that the application of a more sensitive method, such as a radioimmunoassay, would have detected specific or cross-reacting antibodies to dextran in our patients. However,

signs of classical pathway activation were not observed. Furthermore, it is well established that native dextrans activate the alternative pathway in vitro (Pillemer et al., 1955; Hänsch et al., 1979). Further references are given by Ring (1980).

Analysis of the complement profile in consecutive samples from a child with meningococcal septicemia showed that at the beginning of the infection C4 was normal and that C3 and factor B levels were low, a combination suggesting activation of the alternative pathway independent of classical pathway components (VII).

McCabe (1973) reported a significant reduction of C3 levels in patients with Gram-negative bacteremia. The lowest C3 levels were found in connection with fatal outcome. In another study of the same subject, Fearon et al. (1975) found depression of C3 to C9 with normal levels of C1, C2 and C4 in samples from patients with Gram-negative bacteremia complicated with shock. Activation of both the classical and the alternative pathway in meningococcal disease has been reported by Greenwood et al. (1976). However, it is not clear if the observed classical pathway activation was due to specific antibodies or was a direct effect of lipid A, which is probably not dependent of antibodies (Morrison & Kline, 1977).

The low C1q levels observed in our investigation (VII) may have been a consequence of binding of C1q to bacterial products in the circulation without activation of C1 (Loos et al., 1974). This interpretation seems likely, as no certain consumption of C4 was found in the samples obtained during the disease. The pericarditis and pleurisy diagnosed on the third day of illness may be manifestations of a complication due to deposition of immune complexes in the tissues. As C4 belongs to the group of rather slow reacting acute phase proteins (Johansson et al., 1972), a masked consumption of classical pathway components from the third day on owing to an accelerating acute phase reaction can therefore not be ruled out.

In a study of Dengue hemorrhagic fever, Bokisch et al. (1973) found reduced serum levels of C3, C4, C5 and factor B in severe cases complicated with shock. The fall in the serum concentration of C3 coincided with the onset of shock. It was questioned whether the anaphylatoxins C3a and C5a could be responsible for the shock symptoms.

The pharmacological effects of the anaphylatoxins are histamine release, vasoconstriction and enhancement of vascular permeability within the microcirculatory system and chemotaxis (Mahler et al., 1975; Chenoweth & Hugli, 1978; Hugli & Müller-Eberhard, 1978). Both anaphylatoxins are extremely potent, and even minute (ng) quantities are sufficient to produce their biological effects (Müller-Eberhard et al., 1972). The spasmogenic activity of C3a and C5a is abolished by the anaphylatoxin inactivator by removal of the C-terminal amino acid arginine (Bokisch & Müller-Eberhard, 1970). Support for the assumption that C3a and C5a participate in the development of shock has been given by Corbin et al. (1976). Using an assay for the anaphylatoxin inactivator they found significantly lowered levels of the inactivator in patients with Dengue hemorrhagic fever and dextran shock.

Since efficient control of complement derived anaphylatoxins is presumably essential for maintenance of a stable circulation, a reduction in the anaphylatoxin inactivator activity might contribute to the development of the clinical shock syndrome.

OTHER ACTIVATION MECHANISMS

(VIII)

Effects of granulocyte collagenase and elastase on the complement components C3 and C5

Highly purified preparations of C3 and C5 were incubated with the proteolytic enzymes, collagenase and elastase, prepared in a highly purified state from human neutrophilic granulocytes.

C3 was purified according to the method of Nilsson and Müller-Eberhard (1965) and when tested against anti-whole human serum it reacted with one precipitation arc. The preparation was devoid of C5 and plasminogen and was stable on incubation at 37°C for 30 minutes. Crossed immunoelectrophoresis revealed no C3b. Reference C3b was prepared by short time trypsinization of isolated C3 (Bokisch et al., 1969) and by treatment of isolated C3 with purified fluid phase C4 $\bar{2}$.

C5 was purified according to Nilsson and Müller-Eberhard (1965). The isolated protein reacted with one precipitate when tested against anti-whole human serum and was unconverted after 30 minutes at 37°C. A C5b-like fragment was prepared by treatment of purified C5 with trypsin, as described by Cochrane and Müller-Eberhard (1968).

The collagenase used in the experiments was prepared from human granulocytes harvested by leukapheresis of the peripheral blood from a patient with chronic myeloid leukemia. The granule extract was passed through Sephadex G 75, Bio Rex 70 and collagen-Sepharose columns and the preparation appeared homogenous when tested on electrophoresis in agarose and polyacrylamide gels. Two electrophoretic variants of the enzyme were found and both were inhibited by α_1 -antitrypsin and α_2 -macroglobulin (Ohlsson & Olsson, 1973; Ohlsson, 1975).

For the preparation of elastase, leukocytes were obtained by leukapheresis. The extract from granules was filtered through Sephadex G 75, ϵ -Ahx-Sepharose (ϵ -aminocaproate-Sepharose) and elastine-Sepharose. The elastase preparation was eluted as three isoenzymes and was pure, as judged from agarose and polyacrylamide gel electrophoresis. The preparation used was inhibited by α_1 -antitrypsin and α_2 -macroglobulin (Ohlsson & Olsson, 1974; Ohlsson, 1975).

Collagenase. When collagenase was added to normal human serum in amounts not exceeding the inhibitory capacity of the plasma protease inhibitor α_1 -antitrypsin, crossed immunoelectrophoresis and immunoelectrophoresis, respectively, showed no conversion of C3 or C5. Increasing of the amounts of collagenase resulted in fragmentation of C3 to a fragment with the same electrophoretic mobility as the reference C3b. Serum C5 resisted the attack by collagenase even when the enzyme was used in high concentrations.

On incubation of purified C3 with collagenase (molar ratio 13:1), most of the C3 was converted to a faster migrating fragment with the same electrophoretic mobility as the reference C3b. As in the serum experiments, collagenase had no effect on C5 when tested on a purified preparation of the protein.

Elastase. In amounts not large enough to exceed the inhibitory capacity of α_1 -antitrypsin, addition of elastase did not cause cleavage of C3 or C5 in normal serum. On amounts saturating the protease inhibitors, C3 was converted to a fragment with the electrophoretic mobility of C3b. Immunoelectrophoresis showed cleavage of C5 and the appearance of a fragment with the mobility corresponding to that obtained by tryptic digestion of purified C5.

Purified C3 incubated with elastase (molar ratio 7:1) resulted in partial conversion to a fragment of C3b mobility, and purified C5 incubated with elastase in the same ratio resulted in conversion to a fragment with an electrophoretic mobility identical with that observed in serum incubated with elastase.

DISCUSSION

Complement activation leads to formation of several fragments which, together with products from other plasma protein systems, such as the coagulation and kinin systems, participate in the inflammatory process (cf Ryan & Majno, 1977).

The pathognomonic features of inflammation are increased vascular permeability and the infiltration of inflammatory cells. In the early stages of inflammation the neutrophil granulocytes predominate. The accumulation of cells at the site of inflammation is mainly the result of chemotaxis.

Activation of the complement system, either by the classical or

by the alternative pathway, generates the chemotactic principle C5a (Chenoweth & Hugli, 1978; Fernandez et al., 1978). Chemotactic activity has also been ascribed to the C5b67 complex (Ward et al., 1965); some investigators consider the observed activity the result of contamination with C5a (Hugli & Müller-Eberhard, 1978).

At the site of inflammation, the most important function of the polymorphonuclear cell is phagocytosis. The phagocytotic process is accompanied by leakage of a variety of enzymes, among them collagenase and elastase.

Proteolytic cleavage of human C3 by leukocyte enzymes derived from lysosomal lysates has been reported earlier (Ward & Hill, 1970; Taubman et al., 1970). At that time the proteases responsible for the C3 degradation were not defined, and the fragments of C3 formed were not characterized. Chemotactic factors have been generated from C5 by proteolytic digestion with trypsin (Cochrane & Müller-Eberhard, 1968).

In the present investigation (VIII) we studied the effects of highly purified leukocyte collagenase and elastase on the complement components C3 and C5, in serum and as highly purified components. It was found that both collagenase and elastase produced a fragment of C3 with the same electrophoretic mobility as C3b. C5 was cleaved only by elastase.

It has since been reported by other investigators who used purified components that human leukocyte elastase induced a highly selective cleavage of C3. The major C3 fragmentation product functioned like a C3b analogue causing factor B cleavage in a test system containing factor B, factor D and Mg^{++} . A fragment formed, which reacted with anti-C3a, was shown to lack the C-terminal amino acid arginine and did not possess the spasmogenic activity of C3a. Prolonged digestion or use of higher elastase concentrations resulted in degradation of the molecule into several smaller fragments (Taylor et al., 1977).

Human leukocyte collagenase and elastase are, when unchecked by the plasma protease inhibitors, fully capable of activating complement at a single component level. Thanks to the positive feedback mechanism of the alternative pathway, the C3b fragments formed can accelerate the turnover of C3 in serum with a de novo production of C3a and C3b.

GENERAL SUMMARY

I. Complement components were analysed in acute pneumococcal otitis media in children. Two groups of patients were studied: one with a recurrence of the disease; the other with a primary attack. The most interesting findings were low serum levels of C1q in the group with a recurrence. In this group the amount and frequency of abnormal C1 subcomponent complexes (C1r-C1s) was higher than in the other. The mechanism responsible for generation of the C1r-C1s complexes and its reflection of C1 dysfunction is discussed.

II. A rapid two-step procedure for purification of C-reactive protein is described. The method utilizes the difference in charge of C-reactive protein in the presence and absence of Ca^{++} and the different affinity to DEAE-cellulose following the change of charge.

III. The C1 subcomponent complexes (C1qrs, C1r-C1s, C1r-C1s-C1 IA) appearing on activation of the classical pathway were studied with crossed immunoelectrophoresis. The following C1 activators were used: proteolytic enzymes (C1r, C1s, trypsin and plasmin), immune complexes, aggregated IgG and C-reactive protein complexed with protamine sulphate. A dissociation in serum of macromolecular C1 (C1qrs) into C1q and C1r-C1s-C1 IA complexes was shown. C1r-C1s could not be generated in vitro with the C1 activators used. It was found that C1r-C1s complexes formed in vivo contained C1r and C1s in the proenzyme form.

IV. Addition of activated C1s to normal serum resulted in cleavage of C3 and factor B, but not in EDTA-plasma or in serum deficient in C4 indicating that the C42 complex triggers off the positive feedback mechanism of the alternative pathway. The findings lend no support to the existence of a previously reported "C1 bypass mechanism" which was claimed to activate the complement at the C3 level and bypass the classical complement components C4 and C2.

V. Activated properdin was shown to cleave purified C3. The cleavage was not dependent of divalent cations, it was not in-

hibited by soyabean trypsin inhibitor and it was abolished on addition of anti-properdin prior to C3. The role of properdin in the alternative pathway is discussed, as is the possibility of different species of properdin serving different purposes.

VI. The complement profile in dextran and Gram-negative shock was studied. The alternative pathway was activated; no definite signs of classical pathway activation were detected with the methods used.

VII. Cleavage C3 and C5, both in serum and as purified components, by elastase prepared from human leukocytes was shown. Purified leukocyte collagenase caused fragmentation of C3 but not of C5. The findings are discussed in relation to the inflammatory reaction.

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